Inhalation Exposure of Rats to Asphalt Fumes Generated at Paving Temperatures Alters Pulmonary Xenobiotic Metabolism Pathways without Lung Injury

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Asphalt fumes are complex mixtures of various organic compounds, including polycyclic aromatic hydrocarbons (PAHs). PAHs require bioactivation by the cytochrome P-450 monooxygenase system to exert toxic/carcinogenic effects. The present study was carried out to characterize the acute pulmonary inflammatory responses and the alterations of pulmonary xenobiotic pathways in rats exposed to asphalt fumes by inhalation. Rats were exposed at various doses and time periods to air or to asphalt fumes generated at paving temperatures. To assess the acute damage and inflammatory responses, differential cell counts, acellular lactate dehydrogenase (LDH) activity, and protein content of bronchoalveolar lavage fluid were determined. Alveolar macrophage (AM) function was assessed by monitoring generation of chemiluminescence and production of tumor necrosis factor-α and interleukin-1. Alteration of pulmonary xenobiotic pathways was determined by monitoring the protein levels and activities of P-450 isozymes (CYP1A1 and CYP2B1), glutathione *S*-transferase (GST), and NADPH:quinone oxidoreductase (QR). The results show that acute asphalt fume exposure did not cause neutrophil infiltration, alter LDH activity or protein content, or affect AM function, suggesting that short-term asphalt fume exposure did not induce acute lung damage or inflammation. However, acute asphalt fume exposure significantly increased the activity and protein level of CYP1A1 whereas it markedly reduced the activity and protein level of CYP2B1 in the lung. The induction of CYP1A1 was localized in nonciliated bronchiolar epithelial (Clara) cells, alveolar septa, and endothelial cells by immunofluorescence microscopy. Cytosolic QR activity was significantly elevated after asphalt fume exposure, whereas GST activity was not affected by the exposure. This induction of CYP1A1 and QR with the concomitant down-regulation of CYP2B1 after asphalt fume exposure could alter PAH metabolism and may lead to potential toxic effects in the lung. *Key words:* **alveolar macrophage, asphalt fumes, CYP1A1, CYP2B1, cytochrome P-450, glutathione *S*-transferase, lung injury, NADPH:quinone oxidoreductase, pulmonary inflammation, xenobiotic pathways.** *Environ Health Perspect* **111:1215–1221 (2003). doi:10.1289/ehp.5740 available via *http://dx.doi.org/* [Online 24 February 2003]**

Approximately half a million workers are exposed to asphalt fumes in the United States. The main use of asphalt is for road paving, and the major routes for asphalt fume exposure are pulmonary inhalation and dermal adsorption. Asphalt fumes are complex mixtures of aerosols and vapors that contain various organic compounds, such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and heterocyclic compounds containing nitrogen, oxygen, or sulfur (King et al. 1984). Because of the presence of PAHs in the asphalt fumes, the potential exposure to carcinogens is a health concern for exposed workers.

During road-paving operations, the presence of low levels of total PAHs, including benzo[*a*]pyrene (BaP), in the paving asphalt fumes has been demonstrated through environmental (Monarca et al. 1987) and personal (Watts et al. 1998) monitoring. Exposure of road pavers to these asphalt fume–associated PAHs was supported by the enhanced level of urinary 1-hydroxypyrene (Jarvholm et al. 1999). Some studies have reported a small genotoxic effect in road pavers caused by their occupational exposure to asphalt fumes (Burgaz et al. 1998; Fuchs et al. 1996). In contrast, substantial genotoxic damage was demonstrated in peripheral mononuclear blood cells of roofers (Fuchs et al. 1996). Epidemiologic evaluation showed relatively lower risks for road pavers than for roofers in developing all types of cancers, including lung, stomach, and bladder cancer (Partanen and Boffetta 1994). The difference in cancer risk for pavers and roofers is probably due to the PAH content in the asphalt fumes, which depends on the generating temperature of the fumes. Indeed, paving asphalt fumes are generated at much lower temperatures than are fumes from asphalt roofing applications, and there is a correlation between mutagenic activity and the amount of three- to seven-ring PAHs in these asphalt fumes (Machado et al. 1993). In light of these studies, there is interest in understanding the effects of asphalt fumes on metabolic pathways involved in activation or deactivation of PAHs in the lung.

The carcinogenic effect of PAHs is closely linked to the activity of the cytochrome P-450 monooxygenase system. Pulmonary P-450 activities are composites of numerous specific isozymes, including constitutive CYP2B1 and PAH-inducible CYP1A1 (Guengerich et al. 1982). The inducible CYP1A1 can metabolize PAHs to form carcinogenic metabolites, which may lead to DNA damage (Burke et al. 1985; Godden et al. 1987; Lacy et al. 1992). Repression of CYP2B1 has been demonstrated in some pneumotoxin-induced lung injuries—for example, in response to 1-nitronaphthalene or ipomeanol (Verschoyle and Dinsdale 1990; Verschoyle et al. 1993). However, this toxin-induced injury was not affected by the induction of CYP1A1, suggesting that alteration of CYP2B1 may result in a pulmonary-specific toxic response. In addition to phase I P-450 systems, various phase II enzymes, including glutathione *S*-transferases (GSTs) and NADPH:quinone oxidoreductase (QR), play important roles in xenobiotic metabolism (Jaiswal 1994; Prestera et al. 1993). Both the induction of CYP1A1 and the lack of GST have been demonstrated to play a role in cancer susceptibility of smokers and coke-oven workers (Bartsch et al. 1999). On the other hand, QR is known to be able to reduce the binding of quinone to DNA and proteins, thus offering a protective effect against genotoxicity (Joseph and Jaiswal 1994). Because the lung is the major target organ for the airborne environmental toxins, exposure to such agents may significantly modulate metabolic pathways and lead to toxic/carcinogenic effects.

A recent epidemiologic study has reported that asphalt fume exposure did not cause significant changes in lung function or pulmonary symptoms among road pavers (Gamble et al. 1999). Previous studies carried out in our laboratory have shown that exposure of rats by intratracheal instillation to paving-temperature asphalt fume condensate, collected at the top of an asphalt storage tank using a cold trap, did not cause lung inflammation or cellular damage and did not alter macrophage function (Ma et al. 2000). However, intratracheal exposure to asphalt condensate significantly induced CYP1A1 in the lung (Ma et al. 2002). These studies show that the major pulmonary effects of asphalt fumes, which lack a particulate component, are not on macrophage-mediated

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inflammatory responses but are on the metabolic enzyme systems that are critical in handling inhaled chemical substances. However, levels of asphalt fume condensate administered by intratracheal instillation were very high, and the acute effects of asphalt fumes on the lung after inhalation exposure have not been investigated. One critical factor in designing an asphalt exposure study is to carry out the inhalation exposure using asphalt fumes generated under road-paving conditions. Such a system would allow characterization of both the inflammatory effects and the modulation of the metabolic enzyme systems resulting from paving asphalt fume exposure. For this reason, an inhalation exposure system has been developed and characterized in our laboratory (Wang et al. 2001).

At present time, a threshold limit value (TLV) for asphalt fumes of 0.5 mg/m^3 (8-hr time-weighted average) is recommended by the American Conference of Governmental Industrial Hygienists (ACGIH 2002). The current National Institute for Occupational Safety and Health (NIOSH) recommended exposure limit (REL) for asphalt fume exposure is 5 mg/m^3 as a ceiling value, which is a concentration not to be exceeded at any time during the workday (NIOSH 2002). However, a permissible exposure limit for asphalt fumes has not yet been promulgated by the Occupational Safety and Health Administration. Because both ACGIH TLV and NIOSH REL values are simply recommendations, workplace exposures to asphalt fumes may exceed these levels and may be of occupational concern. In the present study, we report the effects of inhaled asphalt fumes generated at paving temperatures on pulmonary damage and inflammation and on the metabolic pathways of the lung involving both phase I and phase II enzymes.

Materials and Methods

Asphalt inhalation exposure system. The asphalt fume generation and inhalation exposure systems have been described previously (Wang et al. 2001). Briefly, road paving asphalt was preheated to 170°C in an oven, transferred to a reservoir (at 170°C), and passed through a heated pipe and onto a heated plate with temperature maintained at 150°C at the inlet and 120°C at the outlet. These simulated asphalt fume generation temperatures are typical of those reported in the field during asphalt road paving. Humidified and temperature-controlled air was blown across the plate to mix with asphalt vapor. The mixture was then transported through a heated pipe into the animal inhalation-exposure chamber. Teflon filters 37 mm in diameter and with 0.45-μm pore size were used for gravimetric analysis of the fumes in the exposure chamber. Teflon filters were backed up with an XAD-2 sorbent tube (SKC Inc., Eighty Four, PA) and a charcoal sorbent tube to collect medium- and small-molecular-weight chemicals, and these samples were used for chemical analysis of the fume generator output as described previously (Wang et al. 2001). The filters were weighed immediately after the sampling period ended, and the fume concentration was determined. In the present study, rats were exposed to asphalt fumes generated at paving temperatures for various exposure time periods. The calculated total asphalt fume exposure (milligrams per hour per cubic meter) for each treatment group (Table 1) was calculated as the product of the asphalt fume concentration (milligrams per cubic meter) and the total exposure time (hours per day × days).

Treatment of animals. Female Sprague-Dawley rats [Hla:(SD)CVF] from Hilltop Lab Animals (Scottdale, PA), monitored free of endogenous viral pathogens, parasites, mycoplasmas, *Helicobacter*, and cilia-associated respiratory bacillus and weighing about 150 g (~5 weeks old) at arrival, were used for all experiments. The rats were kept in cages ventilated with filtered air on Alpha-dri virgin cellulose chips (ALPHA-dri, Shepherd Specialty Papers; T.R. Last Company, Gibsonia, PA) and hardwood Beta-chips (Beta Chip, Northeastern Products Corp.; Famers Delight Company, Grafton, WV) as bedding and were provided HEPA-filtered air, autoclaved Prolab 3500 diet (PMI Nutrition International, Inc.; T.R. Last Company), and tap water *ad libitum* under controlled light-cycle (12:12-hr light:dark cycle) and temperature (22–24°C) conditions. The facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, specific pathogen-free, and environmentally controlled. The rats were acclimated for 2 weeks in exposure chambers before use. Rats were exposed by inhalation to asphalt fumes under conditions as described in Table 1 and sacrificed the next day. Control animals were exposed to air that was regulated at the same flow rate, temperature, and humidity as in the asphalt exposure experiment.

Isolation of alveolar macrophages. Animals were anesthetized with sodium pentobarbital (0.2 g/kg body weight) and exsanguinated by cutting the renal artery. Alveolar macrophages (AMs) were obtained by pulmonary lavage with a Ca^{2+}-, Mg^{2+}-free phosphate-buffered medium (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH 7.4). A total of 80 mL of bronchoalveolar lavage fluid (BALF) was collected from each animal and was centrifuged at 500 × *g* for 5 min. Cell pellets were combined, washed, and resuspended in a HEPES-buffered medium (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 5.5 mM glucose, and 1.0 mM $CaCl_2$; pH 7.4). The acellular supernate from the first lavage (6 mL) was saved separately from the subsequent lavages for further analysis of lactate dehydrogenase (LDH) activity and protein content. Cell counts and purity were measured using an electronic cell counter equipped with a cell-sizing attachment (Coulter Multisizer II with a 256C channelizer; Coulter Electronics, Hialeah, FL). Polymorphonuclear leukocytes (PMNs) and AMs were differentiated by their characteristic cell volumes (Castranova et al. 1990).

AM cultures. Lavage cells were resuspended in Eagle minimum essential medium (EMEM; BioWhittaker, Walkersville, MD) containing 1 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% heat-inactivated bovine serum. Aliquots of 1 mL, containing 1 × 10^6 AMs, were added to a 24-well tissue culture plate and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 2 hr. The nonadherent cells were then removed with three washes of EMEM, and the adherent macrophage-enriched cells were used for all AM culture studies. AMs were cultured in fresh EMEM for an additional 24 hr. The macrophage-conditioned media were collected and centrifuged, and the supernates were saved. The conditioned media were stored in aliquots at –80°C for further analysis of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1).

Protein, LDH activity, and chemiluminescence determination. The acellular LDH activity in BALF was monitored and used as a cytotoxicity index. LDH activity was determined by measuring the formation of NADH, which was monitored spectrophotometrically at 340 nm, using Roche Diagnostic Systems (Indianapolis, IN) reagents and procedures, on an automated Cobas FARA II analyzer (Roche Diagnostic Systems, Montclair, NJ). The protein content in the acellular BALF, used as a marker for air–blood barrier damage, was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) following the manufacturer's protocol.

Chemiluminescence (CL) generated by AMs was determined using an automated luminometer (Berthold Autolumat LB 953, Wallac, Inc., Gaithersburg, MD). Rat AMs (1 × 10^6/mL) were preincubated at 37°C for 10 min in HEPES-buffered medium, containing 0.08 μg/mL luminol. Zymosan (2 mg/mL) was added to the preincubated AM sample, and

Table 1. Total asphalt fume exposure.

Exposure concentration (mg/m^3)	Exposure time	Total exposure concentration (mg hr/m^3)
52.9	1 hr	52.9
58.8 ± 5.6	6 hr	352.9 ± 33.6
10.4 ± 1.4	3.5 hr/day, 5 days	182 ± 24.5
23.6 ± 3.3	3.5 hr/day, 5 days	413.5 ± 57.7
16.7 ± 1.9	6 hr/day, 5 days	547 ± 57.9
24.4 ± 2.0	6 hr/day, 5 days	732 ± 60
57.8 ± 3.0	6 hr/day, 5 days	1,734 ± 90

CL production was immediately measured for 15 min. CL generated from AMs was plotted versus time, and the area under the curve was integrated to give the total CL in cpm/0.75 × 10^6 AMs/15 min.

Cytokine assays. TNF-α in the macrophage-conditioned medium was determined using an enzyme-linked immunosorbent assay (Biosource International, Inc., Camarillo, CA) according to the manufacturer's instructions. The IL-1 activity in the macrophage-conditioned medium was determined using thymocyte proliferation assay according to the method described previously (Kang et al. 1992). The amount of IL-1–like activity in the tested macrophage supernates was expressed as disintegrations per minute. Antibodies of anti-rat IL-1α and anti-rat IL-1β (Endogen, Cambridge, MA) were used to confirm IL-1 activity measured by the thymocyte proliferation assay. These antibodies completely neutralized the activity in the macrophage-conditioned supernate, thus indicating that the increase in thymocyte proliferation was due to the presence of IL-1 in the macrophage-conditioned culture media.

Isolation of microsomes. Rats were anesthetized with pentobarbital sodium (0.2 g/kg body weight), and the heart and lungs were removed. The lungs were perfused with saline solution to remove blood cells. The lung tissue was chopped four times with a McIlwain tissue chopper (Mickle Engineering Co., Gomshall, Surrey, UK) set at 0.5-mm slice thickness. The minced lungs were suspended in 4× lung weight of ice-cold incubation medium (145 mM KCl, 30 mM Tris-HCl, 1.9 mM KH_2PO_4, 8.1 mM K_2HPO_4, and 3 mM $MgCl_2$; pH 7.4) and homogenized, using a Teflon–glass Potter-Elvejhem homogenizer (Talboys Engineering Corp., Emerson, NJ) for 16 complete passes. The microsomal fraction of the tissue homogenate was obtained by differential centrifugation as described previously (Miles et al. 1996). The microsomal pellet was resuspended in incubation medium at a tissue concentration of 1 g/mL. The protein concentration of the microsomal fraction was determined using a BCA protein assay kit (Pierce).

Enzyme assays. NADPH cytochrome *c* (P-450) reductase activity was determined in lung microsomal suspensions (0.1 mg protein/mL) by measuring the rate of reduction of cytochrome *c* (final concentration, 40 μM) monitored at 550 nm, using a Gilford Response II spectrophotometer (CIBA-Corning Diagnostics Corp., Oberlin, OH) in the split-beam mode. The extinction coefficient used was 18.7 $mM^{-1}cm^{-1}$ (Masters et al. 1967), and results were expressed as nanomoles of cytochrome *c* reduced per milligram of microsomal protein per minute. The suspensions contained 2.2 mM KCN to prevent any oxidation of reduced cytochrome *c* by potential mitochondrial contamination.

The activities of CYP2B1 and CYP1A1 were determined by measuring the *O*-dealkylation of 7-pentoxyresorufin (PROD) and 7-ethoxyresorufin (EROD), respectively, in microsomal suspensions, containing 0.1 mg/mL microsomal protein in an incubation medium buffered at pH 7.6 according to the method of Burke et al. (1985). The reaction mixture contained 5 μM 7-pentoxyresorufin or 3 μM 7-ethoxyresorufin (Sigma Chemical Co., St. Louis, MO) and was initiated by addition of 0.48 mM NADPH at 37°C. The activities of PROD and EROD were measured by monitoring the formation of resorufin spectrofluorometrically at an excitation wavelength of 530 nm and emission of 585 nm (model LS-50 Luminescence Spectrometer; Perkin-Elmer Corp., Norwalk, CT) at various times. The results were expressed as picomoles of resorufin formed per minute per milligram of microsomal protein.

GSTs are a group of enzymes catalyzing the conjugation of reduced glutathione (GSH) with a wide variety of electrophilic compounds. GST activity was determined using the generic GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma) according to the method of Habig et al. (1974). The final reaction mixture contained 1 mM CDNB and 1 mM GSH in 0.1 M potassium phosphate buffer (pH 6.5). The reaction was started by the addition of lung-soluble supernate (cytosol), and the increase in absorbance was measured spectrophotometrically at 340 nm using a Shimadzu UV-2401PC spectrophotometer (Shimadzu, Columbia, MD).

The cytosolic QR activity was determined by monitoring dicumarol-inhibitable QR activity, using 2,6-dichlorophenolindophenol (Sigma Chemical Co., final concentration 40 μM) as the substrate (Benson et al. 1980). The loss of optical density at 600 nm was recorded for 10 min at 37°C using a Shimadzu UV-2401PC spectrophotometer. In another set of experiments, 10 μM dicumarol, an inhibitor of cytosolic QR, was added to the reaction mixture to assure that the disappearance of the substrate was due to a cytosolic QR enzyme reaction.

Immunochemical analysis. Lung microsomal proteins, at equivalent amounts (20 μg for CYP2B1 and 75 μg for CYP1A1) of each sample, were subjected to discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) using 4–12% gradient Tris-glycine gels (Novex, San Diego, CA) for separation. After electrophoresis, the proteins were transferred by electroblotting from the gel to a nitrocellulose membrane, according to the manufacturer's instructions (Novex). CYP1A1 and CYP2B1 were detected by immunochemical reaction using commercial kits purchased from Amersham (Piscataway, NJ). The proteins bound to the nitrocellulose membrane were reacted with a rabbit polyclonal antibody, prepared against rat microsomal CYP1A1 or CYP2B1 at 4°C overnight. The membrane was washed with 20 mM Tris-HCl (pH 7.4) buffer containing 0.137 M NaCl and 0.1% Tween 20 (TBS-T) three times and then incubated with an anti-rabbit immunoglobulin (Ig)-biotinylated secondary antibody (1 hr, room temperature), followed by TBS-T washing and a 20-min incubation with a streptavidin–horseradish peroxidase conjugate that binds to the biotinylated secondary antibody. The immunocomplexes were determined by the enhanced CL detection method (Amersham Pharmacia, Piscataway, NJ). The intensity of the protein band on the X-ray films was scanned using the Stratagene Eagle Eye II (La Jolla, CA) with Eagle Sight software. After scanning, the density was measured using Image Quant 5.1 software from Amersham.

Immunofluorescence for CYP1A1. The lungs of asphalt-exposed rats were rapidly removed; the right mainstem bronchus was ligated at the mainstem bronchus, the right lung was removed, and the left lung was airway perfused with 3 mL 10% neutral buffered formalin. The left lung was trimmed within 24 hr of necropsy, processed overnight, and embedded in paraffin the next morning. Immunofluorescence was used to localize CYP1A1 within the lung.

For immunofluorescent staining for CYP1A1, paraffinized sections were placed on Silane-treated slides (ProbeOn Plus; Fisher Scientific, Pittsburgh, PA) and deparaffinized, and antigenicity was retrieved using 0.01 M citrate in a microwave oven. The slides were blocked for nonspecific staining with 5% bovine serum albumin and then incubated with a 1:2 dilution of rabbit anti-CYP1A1 primary antibody (Amersham) overnight at room temperature followed by a 2 hr incubation at 37°C. The slides were then incubated for 2 hr at room temperature with a 1:20 dilution of the secondary antibody, Alexa 488–labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Using these techniques, we obtained lung sections in which CYP1A1 stained green.

Statistical analysis. Results were expressed as means ± SE from at least five different animals. Statistical analyses were carried out with the JMP IN statistical program (SAS, Inc., Cary, NC). The significance of the interaction among the different treatment groups was assessed using analysis of variance and analyzed using the Tukey-Kramer post hoc test. Significance was set at $p < 0.05$.

Results

Acute pulmonary responses. The acute effects of asphalt fumes on pulmonary inflammation and lung injury were monitored. The differential counts of pulmonary lavage cells and the increase in PMN counts were determined as

an index of inflammation. Protein content and LDH activity in acellular first lavage fluid were measured to monitor injury to the air–blood barrier and cell cytotoxicity, respectively. Table 2 shows that acute exposure of rats to asphalt fumes by inhalation did not cause significant neutrophil infiltration into the air space. The amount of total protein and the activity of LDH in the acellular lavage fluid from asphalt fume–exposed rats were also normal compared with the controls. These results suggest that short-term exposure of rats to paving-temperature asphalt fumes did not cause significant pulmonary inflammation or lung injury.

Acute effects on AM activity. Table 3 shows the effects of asphalt fume exposure on the production of reactive oxygen species and proinflammatory cytokines by AMs. The respiratory burst activity of AMs, indicated by the cellular generation of CL in response to zymosan stimulation, and the production of proinflammatory cytokines TNF-α and IL-1 by AMs with or without lipopolysaccharide (LPS) stimulation, were not significantly affected by acute asphalt fume exposure. The results shown in Tables 2 and 3 confirm our earlier intratracheal instillation findings that asphalt condensate collected from fumes generated at road-paving temperatures did not cause damage to the air–blood barrier or cytotoxicity in the lung and did not activate or depress AM function measured as secretion of proinflammatory cytokines or other reactive species (Ma et al. 2000).

Microsomal protein content and cytochrome P-450 reductase activity in lung microsomes. Table 4 shows the effects of asphalt fume inhalation on the microsomal protein content and the NADPH cytochrome *c* reductase activity of the lung. The results indicate that inhalation of asphalt fumes generated at paving temperatures did not significantly alter the total protein level or the NADPH cytochrome *c* reductase activity of lung microsomes.

Alteration of CYP1A1 and CYP2B1 protein levels and activities. The CYP1A1 and CYP2B1 activities in lung microsomes were determined by monitoring the dealkylation of EROD or PROD, respectively. Figure 1 shows the biotransformation of 7-ethoxyresorufin (Figure 1A) and 7-pentoxyresorufin (Figure 1B) to resorufin in microsomal suspensions from rats exposed to clean air or to asphalt fumes generated at paving temperatures. The results show that asphalt fume exposure induced microsomal CYP1A1-specific EROD activity compared with air-exposed controls in a total exposure-dependent manner. In contrast, microsomal CYP2B1-specific PROD activity was progressively reduced as the total asphalt fume exposure increased.

The CYP1A1 and CYP2B1 proteins were fractionated by SDS/PAGE and transferred to a nitrocellulose membrane for Western blot analysis using rabbit polyclonal antibodies specific for CYP1A1 and CYP2B1. As shown in Figure 2A, there was very little CYP1A1 antibody–reactive protein in microsomes obtained from control lung tissues. However, after asphalt fume exposure, there was a marked induction of immunoreactive CYP1A1 protein in the rat lung microsomes. An increase of total asphalt fume exposure from 53 to 1,733 mg hr/m^3 produced an exposure-dependent induction of CYP1A1 protein in lung microsomes (Figure 2B). The rat lung microsomes have relatively high endogenous levels of CYP2B1, as shown in Figure 2C. Figure 2D shows that the constitutive CYP2B1 content was not affected by the asphalt fume exposure at total exposures of 53 and 353 mg hr/m^3 but was significantly attenuated at asphalt fume exposures of 414 mg hr/m^3 or higher. This asphalt fume concentration-dependent reduction of CYP2B1 in lung microsomes was found to correlate with the inhibitory effects of asphalt fume exposure on PROD activity (Figure 1).

Localization of CYP1A1 in asphalt fume–exposed lungs. Figure 3 shows the localization of CYP1A1 in asphalt fume (547 ± 57.9 mg hr/m^3)–exposed rat lung tissue using an immunofluorescence technique. After exposure to asphalt fumes, CYP1A1 is induced and is localized mainly in alveolar septa, nonciliated airway epithelial cells, and endothelial cells, as indicated by the green fluorescence.

GST and QR activity. The effects of asphalt fume exposure on phase II drug metabolism enzymes GST and QR were monitored in the lung cytosolic fraction. Exposure

Table 2. The effects of asphalt fume exposure on pulmonary inflammation and lung injury.

Treatment	PMNs (×10^6)	LDH (U/L)	Protein (mg/mL)
3.5 hr/day, 5 days			
Air control	1.04 ± 0.15	49 ± 5	0.57 ± 0.06
Asphalt (414 mg hr/m^3)	0.79 ± 0.05	56 ± 5	0.50 ± 0.04
6 hr/day, 5 days			
Air control	0.72 ± 0.08	49 ± 4	0.37 ± 0.04
Asphalt (547 mg hr/m^3)	0.79 ± 0.06	60 ± 5	0.40 ± 0.05
6 hr/day, 5 days			
Air control	1.22 ± 0.16	85 ± 4	0.43 ± 0.01
Asphalt (1,733 mg hr/m^3)	0.71 ± 0.07	83 ± 11	0.42 ± 0.03

Rats were exposed to air or asphalt fumes for 5 days, at total asphalt exposures of 414 mg hr/m^3, 547 mg hr/m^3, or 1,733 mg hr/m^3, and sacrificed the next day. Values represent mean ± SE (n = 8).

Table 3. Effects of paving asphalt fume exposure on AM function.

	CL (10^5 counts/min/10^6 cells)		TNF-α (ng/10^6 AMs)		IL-1 (dpm/10^6 AMs)	
Treatment	Resting	Zymosan stimulated	− LPS	+ LPS	− LPS	+ LPS
3.5 hr/day, 5 days						
Air control	ND	ND	388 ± 83	15,038 ± 3,153	906 ± 157	84,504 ± 11,878
Asphalt (182 mg hr/m^3)			382 ± 134	12,522 ± 3,248	851 ± 68	60,798 ± 12,091
3.5 hr/day, 5 days						
Air control	1.41 ± 0.06	0.91 ± 0.12	ND	ND	ND	ND
Asphalt (414 mg hr/m^3)	1.38 ± 0.05	1.21 ± 0.07				
6 hr/day, 5 days						
Air control	1.45 ± 0.05	0.89 ± 0.14	396 ± 61	3,001 ± 1,578	1,756 ± 168	3,509 ± 905
Asphalt (547 mg hr/m^3)	1.60 ± 0.08	2.02 ± 0.43	740 ± 187	5,162 ± 1,993	1,550 ± 201	2,529 ± 496
6 hr/day, 5 days						
Air control	ND	ND	1,507 ± 340	ND	2,011 ± 243	ND
Asphalt (1,733 mg hr/m^3)			3,723 ± 1,111		2,080 ± 378	

ND, not determined. The resting and zymosan-stimulated CL generated by AMs was determined to monitor AM respiratory burst activity. TNF-α and IL-1 production by AMs with or without LPS (0.1 μg/10^6 cells) stimulation was assayed to monitor the effects of asphalt fume exposure on macrophage secretion of proinflammatory cytokines. Values represent mean ± SE (n = 6–8).

Table 4. Microsomal protein content (mg/g lung) and NADPH cytochrome *c* reductase activity (nmol/min/mg of microsomal protein) microsomes isolated from air control and paving asphalt fume–exposed rats.

Treatment	Microsomal protein	NADPH cytochrome *c* reductase activity
3.5 hr/day, 5 days		
Air control	4.38 ± 0.29	ND
Asphalt (414 mg hr/m^3)	4.18 ± 0.20	
6 hr/day, 5 days		
Air control	3.07 ± 0.18	14.15 ± 1.06
Asphalt (547 mg hr/m^3)	3.26 ± 0.19	12.68 ± 0.60
6 hr/day, 5 days		
Air control	4.11 ± 0.41	20.29 ± 4.32
Asphalt (1,733 mg hr/m^3)	4.11 ± 0.23	18.95 ± 0.42

ND, not determined. Microsomes were isolated from air-exposed or asphalt fume–exposed lungs by differential centrifugation, and microsomal protein content was determined. NADPH cytochrome *c* reductase activity was monitored by measuring the amount of cytochrome *c* reduced. Values represent mean ± SE (n = 7–8).

of rats to asphalt fumes generated at paving temperatures did not alter the total cytosolic GST activity, as shown in Table 5, or the protein level of GST-π (data not shown). In contrast, except at the low total asphalt fume concentration of 53 mg hr/m^3, asphalt fume exposure significantly induced cytosolic QR activity compared with air-exposed controls (Table 5).

Discussion

Pulmonary tissue is a major target for various environmental airborne pollutants and chemicals. This study shows that inhalation exposure of rats to asphalt fumes generated at paving temperatures does not cause acute damage to the alveolar air–blood barrier, cytotoxicity, or inflammation, as indicated by acellular BALF levels of protein and LDH activity, PMN infiltration, and CL generation and cytokine production by AMs. In addition, asphalt fume inhalation did not compromise the ability of AMs to respond to zymosan or bacterial LPS *ex vivo*. However, a clear modulation of the pulmonary xenobiotic metabolic pathways was observed in response to acute asphalt fume exposure. Acute asphalt fume exposure produced a chemical effect that altered both phase I and phase II enzyme levels and activities in the lung, including the induction of CYP1A1 and QR and a concomitant reduction of CYP2B1 without affecting GST activity. This alteration of xenobiotic enzymes in the lung may significantly affect PAH metabolism and lead to increased susceptibility of the lung to toxic effects.

Reports concerning the direct pulmonary effects of asphalt fume exposure on road workers are not consistent. Studies have shown that asphalt fume exposure induced subjective symptoms (Norseth et al. 1991) and pulmonary disorders in some pavers (Maizlish et al. 1988; Norseth et al. 1991); however, Gamble et al. (1999) recently reported no consistent association between asphalt fume exposure level and the reduction in lung function or the incidence of symptoms among road workers. The present study shows that exposure of rats to asphalt fumes generated at paving temperatures did not induce infiltration of neutrophils into the air spaces or cause damage to the air–blood barrier or cytotoxicity. In addition, asphalt fume exposure did not activate AMs to generate reactive oxygen species or produce inflammatory cytokines TNF-α and IL-1. Furthermore, it did not significantly affect the ability of AMs to respond to microbial products, measured as oxidant production in response to zymosan or cytokine production in response to LPS. These results are consistent with those of a previous study indicating that exposure of rats by intratracheal instillation with asphalt fume condensate, collected from the top of an asphalt storage tank, did not cause pulmonary damage, induce inflammation, or alter AM activity (Ma et al. 2000). This lack of acute pulmonary responses to asphalt fume exposure suggests that asphalt fumes are probably not processed by AMs in the same manner as are organic compounds adsorbed on particles, for example, diesel exhaust particles (DEP). Indeed, intratracheal instillation of rats with DEP, particles with adsorbed organic compounds, caused lung injury and inflammation, increased basal oxidant and cytokine production, but depressed the ability of AMs to generate oxidant species or produce cytokines in response to subsequent zymosan or LPS treatment (Castranova et al. 2001; Yang et al. 1997, 1999).

Investigation of the potential carcinogenic and/or mutagenic effects of asphalt fume exposure during road paving have yielded inconsistent results. However, roofers exposed to asphalt fumes generated at higher temperatures have consistently exhibited a greater risk for developing lung and stomach cancers than did road pavers (Partanen and Boffetta 1994) and exhibit substantial genotoxic damage in peripheral mononuclear blood cells (Fuchs et al. 1996). Therefore, one might ask whether relatively high-molecular–weight PAHs (i.e., those that might present a cancer risk) are generated at paving temperatures. Chemical analysis of asphalt fumes, produced by our generator, has demonstrated the presence of PAHs (Wang et al. 2001). The present study shows that exposure of rats to asphalt fumes generated at paving temperatures resulted in inhaled PAH levels that were sufficient to alter pulmonary xenobiotic metabolic enzymes, causing induction of CYP1A1 as well as reduction of CYP2B1. However, our previous study demonstrated that exposure of rats by intratracheal instillation to asphalt fume condensate induced pulmonary CYP1A1 without affecting the constitutive CYP2B1 (Ma et al. 2002). The discrepancies between these two studies may be caused by the exposure route and the chemical composition of the asphalt fume—that is, fume condensate versus the whole fume. Because PAHs are the inducers as well as substrates for CYP1A1, the chemical composition of the fumes may play an important role in the

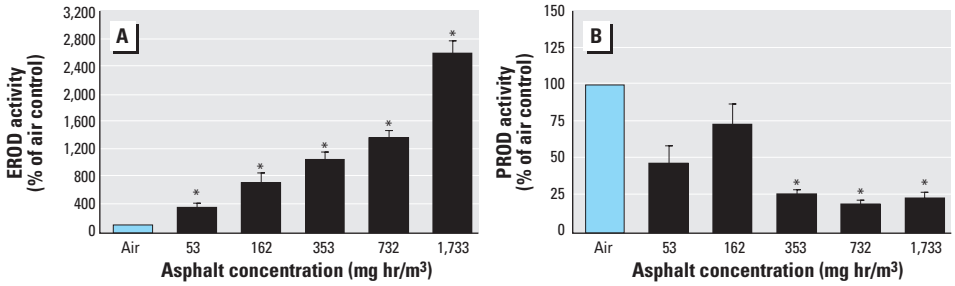

Figure 1. Effects of inhalation exposure of rats to asphalt fumes on CYP1A1 and CYP2B1 activities. Xenobiotic metabolism of EROD (*A*) and PROD (*B*) were monitored in microsomal suspensions by measuring the production of resorufin. Microsomes were purified by differential centrifugation of rat lung homogenates isolated 1 day after exposure to air (control) or asphalt fumes. The *O*-dealkylations of EROD and PROD represent CYP1A1 and CYP2B1 activities, respectively. Values represent mean ± SE (n = 6–8).
*Significantly different from air control group, $p < 0.05$.

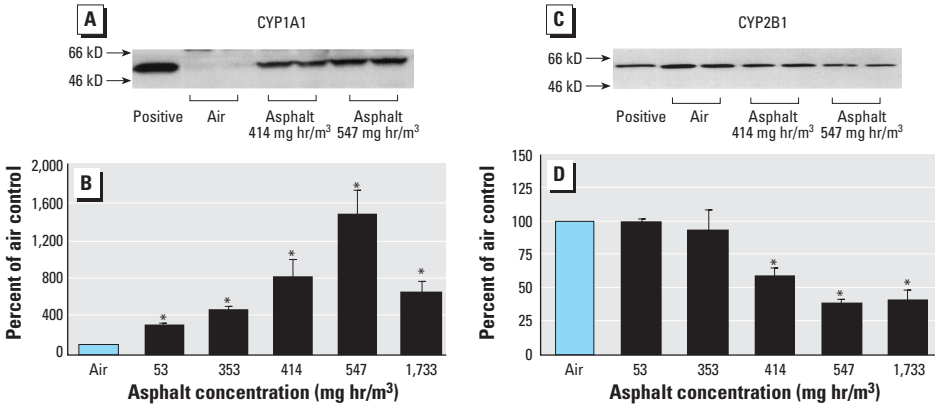

Figure 2. Effects of asphalt fume exposure on CYP1A1 and CYP2B1 levels. The CYP1A1 and CYP2B1 proteins were fractionated by SDS/PAGE and transferred to a nitrocellulose membrane for Western blot analysis, using rabbit polyclonal antibodies specific for CYP1A1 or CYP2B1. Representative Western blots for CYP1A1 (*A*) and CYP2B1 (*C*). Intensities of the protein bands, quantified by densitometry, for CYP1A1 (*B*) and CYP2B1 (*D*), at all asphalt exposure concentrations. Values represent mean ± SE (n = 6–8).
*Significantly different from air control group, $p < 0.05$.

modification of xenobiotic metabolism in the exposed lung. The chemicals contained in the whole fume generated in the laboratory included not only the higher-boiling–point compounds but also the smaller compounds formed in the vapor phase; however, the cold-trap–collected fume condensate may not contain some of the compounds in the vapor phase of the whole fume that may be capable of modulating the metabolic enzymes. This is possible because exposure of mice to naphthalene, a small-molecular–weight component of asphalt fumes, can significantly reduce pulmonary P-450 content and microsomal enzyme activities (Tong et al. 1982). Decreased CYP2B1 has also been shown in the rabbit lung after β-naphthoflavone (β-NF) treatment (Bhagwat et al. 1999) but not in 3-methylcholanthrene (3MC)-exposed rat lungs (Godden et al. 1987), suggesting that alteration of P-450 metabolic pathways is especially substrate dependent. In this light, any change in the fume composition may result in a significant difference in its ability to modify metabolic pathways.

The PAH-mediated increase of CYP1A1 by asphalt fumes is in good agreement with the effects of exposure to other mixed exposure systems, including cigarette smoke (Willey et al. 1997) and DEP (Pott and Heinrich 1990). Cytochrome P-450 isozymes are known for their substrate selectivity. In the case of BaP, CYP1A1 prefers bay-region (7,8 positions) oxidation (Gozukara et al. 1982) and yields metabolites that can be further metabolized to ultimate carcinogens, whereas CYP2B1 catalyzes oxidation of BaP at the 4,5 positions (Gozukara et al. 1982) to yield metabolites that can be easily removed (Smith and Bend 1980). This demonstrates that different isozymes are involved in the regulation of the balance between activation and detoxification pathways of PAH metabolism. Therefore, induction of CYP1A1 with down-regulation of CYP2B1 after asphalt fume exposure may lead to increased production of toxic metabolites of BaP.

There are more than 40 different cell types in the lung. Asphalt fume-induced CYP1A1, as shown by immunofluorescence microscopy, was localized mainly in nonciliated bronchiolar epithelial (Clara) cells, the alveolar septa, and endothelial cells. We found no evidence of proliferation of any specific cell type, but instead observed site-specific induction localized to these areas. These results are consistent with the findings for 3MC-exposed (Keith et al. 1987) or β-NF–exposed rat lungs (Lacy et al. 1992). The localization of CYP1A1 in selected lung cell populations suggests that there may be selective targets in the lung due to metabolic activation of toxic compounds in certain individual cell types.

Asphalt fume exposure did not alter GST but enhanced the QR activity in the lung. These phase II enzymes are known for their detoxification activities. Using cells transfected with plasmids that express elevated levels of selective enzymes individually or in combination, Joseph and Jaiswal (1994) demonstrated that semiquinone, a metabolite of BaP generated after CYP1A1 and NADPH P-450 reductase activation, resulted in distinct BaP–DNA adducts. However, the formation of DNA adducts was significantly reduced by inclusion of a high level of QR activity. These studies clearly demonstrate the importance of keeping these enzymes in balance, and the important role of phase II enzymes in PAH-induced DNA damage. In addition, there is an interactive effect resulting from exposure to mixed PAHs. Pott and Heinrich (1990) have shown in animal studies that inhaled cigarette smoke or DEP contains much less BaP than do fumes from a coke oven or tar pitch but exhibits similar carcinogenic activity. These studies suggest that exposure to mixed PAHs

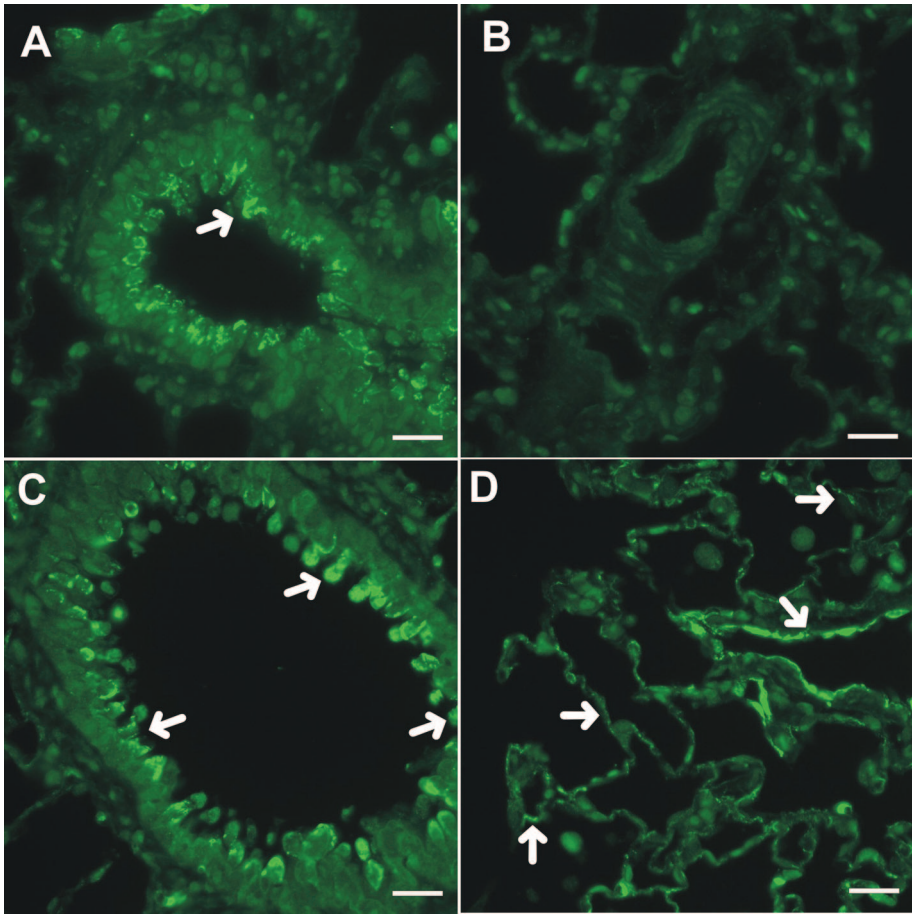

Figure 3. Immunofluorescence micrographs of CYP1A1 localization in air-exposed and asphalt fume–exposed (547 mg hr/m^3) rat lungs. CYP1A1 was labeled with a green fluorochrome (Alexa 488). Green fluorescence indicates the localization of CYP1A1. (*A*) Airways of control rats. (*B*) Alveoli of control rats. (*C*) Airways of asphalt-exposed rats. (*D*) Alveoli and vascular endothelium of asphalt-exposed rats. Arrows point to sites of immunoreactivity. Bars = 20 μm.

Table 5. GST and QR activities in cytosolic proteins isolated from air control and paving asphalt fume–exposed rats.

Treatment	GST activity (nmol/min/mg protein)	QR activity (nmol/min/mg protein)
1 hr		
Air control	184 ± 16	742 ± 39
Asphalt (52.9 mg hr/m^3)	143 ± 8	764 ± 24
3.5 hr/day, 5 days		
Air control	ND	439 ± 39
Asphalt (414 mg hr/m^3)		836 ± 28*
6 hr/day, 5 days		
Air control	369 ± 31	783 ± 28
Asphalt (732 mg hr/m^3)	363 ± 37	1,291 ± 32*
6 hr/day, 5 days		
Air control	ND	592 ± 91
Asphalt (1,733 mg hr/m^3)		1,375 ± 292*

ND, not determined. Cytosolic proteins were isolated from air- or asphalt fume–exposed lungs by differential centrifugation, and cytosol protein content was determined. GST activity was determined by measuring the binding of GSH with CDNB. The QR activity was monitored by measuring the reduction of 2,6-dichlorophenolindophenol by cytosolic protein. Values represent means ± SE (n = 4).
*Significantly different from air control group, $p < 0.05$.

may synergistically induce carcinogenic effects. Boffetta et al. (1997) have shown that genetic cancer susceptibility and DNA adducts in smokers, tobacco chewers, and coke oven workers are related to the up-regulation of a cancer-predisposing gene, *CYP1A1*, and lack of a protective factor, *GSTM1*.

In summary, the present study shows that acute exposure to asphalt fumes generated in the laboratory under road-paving temperatures did not cause alveolar capillary damage or pulmonary inflammation or alter AM activity. However, such exposure significantly altered xenobiotic metabolizing enzymes in the lung, characterized as the induction of CYP1A1 and QR and a down-regulation of CYP2B1. Such alteration in metabolic enzymes would favor PAH activation for the formation of toxic PAH metabolites that might lead to mutagenic/carcinogenic effects in the lung.

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